CLAIMS

1. A process for preparation of a cross linked protein crystals which comprises (a) crystallizing the protein in water with a suitable salt and cosolutes in presence of an organic cosolvent at a temperature ranging between 4^0 to 10^0 C for a period ranging between 5 hr. to 20 days to obtain the crystals of the protein having a cross-section of ranging between 50 to 150 microns,

(b) reacting the crystals of the protein obtained instep (a) with a multifunctional crosslinking agent in the presence of buffer of pH ranging between 3-10 at a temperature ranging between 4^0 to 10^0 C to get the crossed linked protein crystal,

(c) washing the cross linked crystals with reagent capable of removing the excess of cross linking reagent to obtain the washed cross linked protein ,

(d) coating cross linked protein crystals with a suitable surfactant, to obtain the stable product.

2. The method as claimed in claim 1, wherein said protein is an enzyme selected from the group consisting of hydrolases, isomerases, lyases, ligases, transferases and oxidoreductases.

3. A process as claimed in claim 2, wherein said enzyme is a hydrolase or an oxidoreductase.

4. The method as claimed in claim 1 to 3, wherein said hydrolase is selected from the group consisting of amylases, like glucoamylase (amyloglucosidase), alpha amylase, beta amylase.

5. The method as claimed in claim 1 to 4 wherein said oxidase is selected from the group of oxidoreductases consisting of various peroxidases, oxidases, laccases of both plant and microbial origin.

6. The method as claimed in claim 1 to 5, wherein said crystal is a microcrystal of any shape and has a cross-section of 100 microns or less.

7. The method as claimed in claim 1 to 6, where said cross linking reagents used is solvent from a group consisting of glutaraldehyde, starch dialdehyde, Dimethyl-3,3'-dithiobispropionimidate, 2-iminothiolane, n-Succinimidyl-(4-azidophenyl)-1,3-dithiopropionate, Ethyl-4-azidophenyl-1,4-dithiobytryrimidate etc.The concentration of cross linking agent can be 1 to 50 mg per gram of the enzyme crystal.

8. The method as claimed in claim 1 to 7, wherein said surfactant used is anionic, neutral,or cataionic.

9. A method as claimed in claim 1 to 8 wherein the cationic surfactant used is selected from the group consisting of amines, amine salts, sulfonium, phosphonium and quaternary ammonium compounds. like Methyl trioctylammonium chloride (ALIQUAT 336) N,N',N'-polyoxyethylene(10)-N-tallow-1,3-diaminopropane (EDT-20,' PEG-10 tallow), PEI(polyethylene imine) and CTAB (cetyl trimethyl ammonium bromide).

10. A method as claimed in claim 1 9 wherein the anionic surfactant used is selected from the group consisting of linear alkylbenzene sulphonate, alpha-olefin sulphonate, alkyl sulphate, Aerosol T, SDS, alcohol ethoxy sulfate, carboxylic acids, sulfuric esters and alkane sulfonic acids. Examples of anionic surfactants include: TRITON QS-30 (Anionic octyl phenoxy polyethoxyethanol), Aerosol 22 ,dioctyl sulfosuccinate (AOT) ,Alkyl Sodium Sulfate (Niaproof): Type-4 ,Type-8 ,Alkyl (C9-C13) Sodium Sulfates (TEEPOL HB7).

11. A method as claimed in claim 1 to 10 wherein the non-ionic surfactant used is selected from the group consisting of nonyl phenol ethoxylate, alcohol ethoxylate, sorbitan trioleate, non-ionic block copolymer surfactants, polyethylene oxide or polyethylene oxide derivatives of phenol alcohols or fatty acids.

12. A method as claimed in claim 1 to 11 wherein the non-ionic surfactant used is selected from the group consisting of Polyoxyethylene Ethers: 4 lauryl Ether (BRIJ 30) , Tween 80, 23 lauryl Ether (BRIJ 35) ,Octyl Phenoxy polyethoxyethanol (TRITONS): Tx-15 ,Tx-100,Tx-114 , Tx-405 ,DF-16 ,N-57 ,DF-12 ,CF-10 ,CF-54 ,Polyoxyethylenesorbitan: Monolaurate (TWEEN 20), Sorbitan: Sesquioleate (ARLACEL 83) ,Trioleate (SPAN 85) ,Polyglycol Ether (Tergitol): Type NP-4 ,Type NP-9 ,Type NP-35 ,TypeTMN-10,Type15-S-3,TypeTMN-6(2,6,8,Trimethyl-4-nonyloxypolyethylen oxyethanol Type 15-S-40.

13. The method as claimed in claim 1 to 12 wherein said surfactant provides a weight ratio of crosslinked enzyme crystals to surfactant between about 1:1,and about 1:5, preferably between about 1:1 and about 1:2..

14. A method as clamed in claim 1 to 13 wherein the surfactant is carried out by contracting the crosslinked enzyme crystals with surfactant for a period of time between about 5 minutes to 24 hours, preferably between about 30 minutes to 24 hours.

15. The method as claimed in claim 1 to 14 wherein the said buffer used for the CLEC preparation can be 10 to 100 mM of standard acetate, phosphate, citrate or any suitable buffer with a pH in the range of 3-10.

16. The cross linked protein crystal according to claim 1 to 15, wherein the said protein crystal is in a lyophilized form.

17. The cross linked protein crystal as claimed in claim 1 to 16, wherein the said crosslinked enzyme crystal having resistance to exogenous proteolysis, such that said crosslinked enzyme crystal retains at least 91% of its initial activity after incubation for three hours in the presence of a concentration of Protease that causes the soluble uncrosslinked form of the enzyme that is crystallized to form said

enzyme crystal that is crosslinked to lose at least 94% of its initial activity under the same conditions, wherein said crystal is in lyophilized form.

18. The cross linked protein crystal according to claim 1 to 17, which permit said enzyme to act upon the substrate, thereby producing said product in said organic solvent or aqueous-organic solvent mixture.

19. The method as claimed in claim 1 to 18 wherein said organic co solvent used is selected from the group consisting of octanes, diols, polyols, polyethers and water soluble polymers.

20. The method as claimed in claim 1, wherein the organic cosolvent used is selected from the group consisting of toluene, octane, tetrahydrofuran, acetone, pyridine, diethylene glycol, 2-methyl-2,4-pentanediol, poly(ethylene glycol), triethylene glycol, 1,4-butanediol, 1,2-butanediol, 2,3,-dimethyl-2,3-butanediol, 1,2-butanediol, dimethyl tartrate, monoalkyl ethers of poly(ethylene glycol), dialkyl ethers of poly(ethylene glycol), and polyvinylpyrrolidone.

21. A cross linked protein crystal formulation comprising about 10 wt % and about 70 wt % of surfactant, by weight of the final formulation, preferably between about 25 wt % and about 45 wt % of surfactant, by weight of the final formulation.

22. A process as claimed in claim 1 wherein the crystals may be used in an aqueous or organic medium for biotransformations, in an assay, diagnostic kit or biosensor for detecting an analyte, in producing a product such as using crosslinked Peroxidase crystals to produce novel polysaccharides, in separating a substance from a mixture, in therapy and in bioremediation of toxic effluents.

AMENDED CLAIMS

[received by the International Bureau on 06 January 2005 (06.01.05);
original claims 1-22 replaced by new claims 1-21 (4 pages)]

1. A process for the preparation of cross linked enzyme crystals of hydrolases, and oxidoreductases which are solvent tolerant, thermostable and shear resistant, the process comprising the steps of:

(a) crystallizing the enzymes in aqueous buffer with a suitable salts and cosolvents in the presence of surfactants at a temperature ranging between 4^0 to 10^0 C for a period ranging between 5 hr. to 15 days to obtain the crystals of the protein having a particle size ranging between 50 to 150 microns;

(b) reacting the crystals of the enzyme obtained in step (a) with a multifunctional crosslinking agent in the presence of buffer of pH ranging between 3-8 at a temperature ranging between 4^0 to 25^0 C to get the crossed linked enzyme crystal;

(c) washing the cross linked crystals with a reagent that is capable of removing the excess of the said multifunctional cross linking reagent so as to obtain the washed cross linked protein; and

(d) coating cross linked protein crystals with a suitable surfactant, and lyophilizing it to obtain the stable product.

2 The process as claimed in claim 1, wherein said enzymes selected from the group consisting of hydrolases and the said enzyme is a starch hydrolyzing amylase namely glucoamylase.

3. A process as claimed in claim 1, wherein said oxidoreductase enzyme is a plant peroxidase.

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4. The process as claimed in claims 1 to 3 wherein said oxidase is selected from the group of plant peroxidases consisting of Horse radish, Ipomea or Saccharum peroxidases.

5. A process as clamed in claim 1 wherein the crystallizing salt is sulphate of ammonium or sodium either as saturated solution or crystals.

6. A process as claimed in claim 1 wherein the said buffer used for the cross linked glucoamylase preparation is an aqueous buffer of 10mM -0.5M of acetate having a pH of 4.5.

7. A process as claimed in claim 1 wherein the said buffer used for the cross linked peroxidase preparation is an aqueous buffer of 10mM -0.5M phosphate or tris having pH of 6.5-8.0.

8. A process for the preparation of the cross linked protein enzyme crystal as claimed in claim 1, wherein the said co-solvent is an alcohol having a concentration of 1-20% , example 2-methyl,2,4 pentane diol; 2-propanol; 1,5 pentane diol, ethanol, methanol, isoamyl alcohol.

9. A process as claimed in claims 1 to 8, wherein said crystal is a microcrystal of 150 microns or less.

10. A process as claimed in claim 1, wherein the cross linking reagents used is glutaraldehyde, and starch dialdehyde.

11. A process as claimed in claim 1, wherein the said surfactant used is anionic, non-ionic, or cationic.

12. A process as claimed in claims 1 to 11 wherein the cationic surfactant used is cetyl trimethyl ammonium bromide or cetrimide.

13. A process as claimed in claims 1-12 wherein the anionic surfactant used is dioctyl sulfosuccinate Aerosol OT.

14. A process as claimed in claims 1 to 13 wherein the non-ionic surfactant used is selected from the group consisting of alkyl phenol ethoxylate, sorbitan trioleate, sorbitan tristerate . Examples Tween 20, Tween 80 and Triton X-100.

15. A process as claimed in claims 1 to 14 wherein the said surfactant provides a weight ratio of crosslinked enzyme crystals to surfactant between about 1:1,and about 1:5, preferably between about 1:1 and about 1:2 and is in a lyophilized form.

16. The process as claimed in claim 1, wherein the cross linked gulcoamylase is active in 1:1 mixture of water organic solvents n-dodecane; n-hexane; chloroform; and dimethyl sulphoxide.

17. A process as claimed in any of the preceding claims, wherein the said crosslinked enzyme crystal is having resistance to exogenous proteolysis, such that said crosslinked enzyme crystal retains at least 91% of its initial activity after incubation for three hours in the presence of a concentration of Protease that causes the soluble uncrosslinked form of the enzyme that is crystallized to form said enzyme crystal that is crosslinked to lose at least 94% of its initial activity under the same conditions, wherein said crystal is in lyophilized form.

18. The process as claimed in claim 1, wherein the cross linked Peroxidases are active in organic solvents like toluene; 80% dioxane, chloroform; 2-propanol; chloroform; acetone; ethanol; acetonitrile; methnol; and dioxane.

19. A process of continuous generation of glucose solution making use of the cross linked enzyme crystal as claimed in claims 1 to 18, wherein the said cross linked glucoamylase crystals are packed in a jacketed column for the continuous saccharification of starch solution having a concentration of 1-20% preferably 4-10%(W/V) at pH 4.5 and at 60° C with a yield of 110g glucose /L/hour at a residence time of 7.6 min.

20. A process of continuous generation of glucose solution making the cross linked glucoamylase crystal as claimed in claim 19, wherein the said enzyme can also act upon a solution of 1-30%(W/V)of maltodextrin of DE 10-15 preferably 10%(W/V) maltodextrin with a DE of 10-14 at a pH of 4.5, at 60° C thereby producing glucose solution within 1-8 min with a yield of 463 to 714 g/L/h.

21. A process as claimed in claims 1 to 18 wherein the crystals of plant peroxidase especially Horse radish peroxidase produces 2, 4 dimethyl phenol dimmer from monomer dissolved either in 2-propanol or toluene and the catalysis carried out at 50°C for 30 min. in the presence of 30% H_2O_2.